AD_____

Award Number: DAMD17-01-1-0256

TITLE: Structure-Based Approach for Discovery of Small Molecule Inhibitors Targeted at Bcl-2

PRINCIPAL INVESTIGATOR: Shaomeng Wang, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109-1274

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (1 Sep 02-31 Aug 03)

4. TITLE AND SUBTITLE
Structure-Based Approach for Discovery of Small Molecule Inhibitors Targeted at Bcl-2

5. FUNDING NUMBERS
DAMD17-01-1-0256

6. AUTHOR(S)
Shaomeng Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Michigan
Ann Arbor, Michigan 48109-1274

E-Mail: Shaomeng@umich.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Overexpression of Bcl-2 has been observed in 70% of breast carcinomas and the expression levels of Bcl-2 proteins correlate with resistance to a wide spectrum of chemotherapeutic drugs and radiation therapy. In this IDEA grant, we propose an effective structure-based approach to discover small molecule inhibitors of Bcl-2 through structure-based 3D-database search over large chemical databases containing >500,000 structurally diverse, non-peptide, drug-like synthetic compounds or natural products. Using this powerful approach, we have discovered 6 classes of structurally diverse, non-peptidic, drug-like, small-molecule inhibitors of Bcl-2. Our studies also showed that the most promising small-molecule inhibitors of Bcl-2 we have discovered potently bind to Bcl-2 protein, inhibit cell growth and induce apoptosis in breast cancer cells with high levels of Bcl-2 proteins and display good selectivity in cancer cells with low levels of Bcl-2 proteins.

14. SUBJECT TERMS
No subject terms provided.

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction: Bcl-2 is the founding member of the Bcl-2 family proteins and potently inhibits apoptosis in cells. As a potent anti-apoptotic molecule, Bcl-2 contributes to cancer cell progression by preventing normal cell turnover caused by physiological cell death mechanisms. Overexpression of Bcl-2 has been observed in 70% of breast carcinomas. The expression levels of Bcl-2 proteins correlate with resistance to a wide spectrum of chemotherapeutic drugs and radiation therapy. The experimental three-dimensional (3D) structure of Bcl-2 showed that Bcl-2 has a surface binding pocket into which pro-apoptotic proteins such as Bid, Bim and Bad bind. This pocket is essential for the anti-apoptotic function of Bcl-2 since mutations at this site abolished Bcl-2 biological function. Therefore, we hypothesize that non-peptide, drug-like, cell permeable small molecules that bind to this surface pocket of Bcl-2 will block the anti-apoptotic function of Bcl-2 and may restore the normal apoptotic process in cancer cells with Bcl-2 protein overexpression and make these cancer cells more susceptible to conventional chemotherapy or radiation therapy. Designing of small molecule inhibitors targeting Bcl-2 at this crucial binding site represents an attractive approach for the development of a novel therapy for the treatment of breast cancer with Bcl-2 protein overexpression.

In this IDEA grant, we propose an effective structure-based approach to discover small molecules that bind to the Bcl-2 binding pocket. Specifically, we propose to perform structure-based 3D-database search over large chemical databases containing >500,000 structurally diverse, non-peptide, drug-like synthetic compounds or natural products to identify small molecule candidates that can effectively interact with the Bcl-2 binding pocket. Most promising candidate molecules are then tested in appropriate binding and cellular assays to confirm their activity, specificity and mechanism. For the best Bcl-2 inhibitors identified from this project, they will be further evaluated for their anti-cancer activity *in vivo* and their therapeutic potential for the treatment of human breast cancer with high levels of Bcl-2 protein.

Discovery of novel, non-peptidic, cell permeable Bcl-2 small molecule inhibitors represents the first but very exciting step toward the development of a novel cancer therapy targeted at Bcl-2. The success of this project will pave the way for the development of a small molecule drug through modulation of the Bcl-2 function for the treatment of breast and many other forms of cancers with Bcl-2 overexpression, either alone or in combination with conventional chemotherapeutic drugs or radiation therapy.

Body of the report:

Task 1. Molecular modeling, structure-based database searching, and computational docking (**1-30 months**).

During the first 12 months of this project, we have largely accomplished the tasks outlined in **Task 1** of the original proposal.

Task 1.1. Extensive molecular dynamics simulation of Bcl-2 through molecular dynamics simulations.

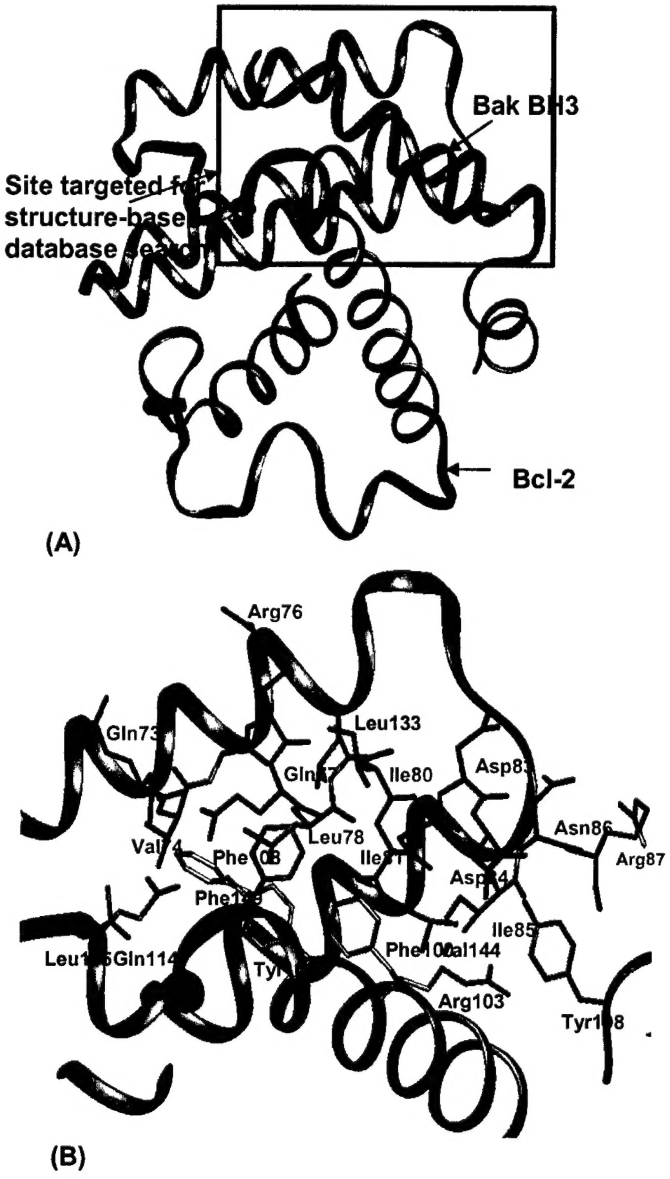
This task is essentially completed.

Structure-based database searching requires an accurate three-dimensional (3D) structure of Bcl-2. The experimental 3D structures of Bcl-2 have been recently determined. However, when we started this project, the experimental Bcl-2 structure was not determined. Fortunately, high resolution experimental 3D structures of Bcl-X_L alone and in complex with a Bak BH3 (Bcl-2 homology domain 3) peptide have been determined. Bcl-2 and Bcl-X_L share a high degree of homology in their amino acid sequences (45% of identity and 56% of similarity). It has been demonstrated that when there is a sequence identity of more than 30% between the target protein (Bcl-2) and the template protein (Bcl-X_L), current computational homology modeling methods, such as MODELLER, can provide an accurate 3D structure for the target protein. Therefore, computational homology modeling was employed to model the 3D structure of Bcl-2 (the target protein) based upon the experimental 3D structural coordinates of Bcl- X_L (the template protein) in this study.

The sequence alignment between Bcl-2 and Bcl-X_L was obtained using the BLAST program, which was used in our homology modeling. Since the Bak BH3 peptide binds to both Bcl-2 and Bcl-X_L with good affinities, the 3D structure of Bcl-2 in complex with the Bak BH3 peptide was modeled based upon the experimental NMR structure of Bcl-X_L in complex with the Bak BH3 peptide. Using the MODELLER program, 10 different models were generated. It was found that these 10 models were very similar, with a root-mean-square deviation (RMSD) within 1 Å for all the non-hydrogen atoms of residues that form the BH3 binding site. To further refine the side chain conformations, the modeled 3D complex structure was extensively simulated through molecular dynamics (MD) simulation in explicit water for 3 ns. Comparison of our modeled Bcl-2 structure with the recently published experimental high-resolution Bcl-2 NMR structure showed that they are essentially the same with respect to both the overall fold and binding site conformation. The root-mean-square deviation (RMSD) is 1.0 Å for all the non-

hydrogen atoms of residues that form the BH3 binding site between the NMR structure and our modeled structure. Thus, computational homology modeling provided us with an accurate 3D structure of Bcl-2 for our structure-based 3D-database searching. The refined structure of Bcl-2 in complex with the Bak BH3 peptide is depicted in **Figure 1**.

Figure 1. Modeled 3D structure of Bcl-2 in complex with Bak BH3 domain based upon the experimental structure of Bcl-XL in complex with Bak-BH3 peptide complex (pdb code: 1BXL). (A) Ribbon representation of the overall Bcl-2 structure in complex with the Bak BH3 peptide. (B). Detailed representation of the binding site. The carbon atoms in the Bak BH3 peptide are in magenta, while the carbon atoms in the Bcl-2 protein are in green, the oxygen atoms are in red and the nitrogen atoms are in blue.



Task 1.2. Structure-based 3D-database searching on four 3D-databases containing more than 650,000 small organic compounds and natural products to identify most promising small molecule inhibitors that effectively interact with the Bcl-2 surface-binding pocket. (**1-30 months**).

During the first 12 months, we have completed the 3D-database searching of the National Cancer Institute's 3D-database of more than 250,000 synthetic organic compounds and natural products.

Since 1955, the National Cancer Institute (NCI) at the National Institutes of Health, USA has conducted extensive testing of materials for possible activity against different forms of cancer. Most of the substances tested have been pure organic compounds. The program examined an extraordinarily eclectic assembly of organic structures. Currently, more than 500,000 compounds have been tested. Of these compounds, about half (250,000 compounds) are classified as "open" compounds, whose structures and biological data can be accessed by the public. Because samples of compounds were needed for testing, it was necessary to develop a large acquisition effort and a repository, both of which are still functioning today. Continual scanning of the chemistry literature allows identification of compounds that are novel and of interest to the program, and the authors are approached for a sample, typically under 100 mg. Currently, the NCI repository has physical samples of about 60% of the registered compounds. Recently, analysis of several large chemical databases showed that the NCI database has by far the highest number of compounds that are unique to it. Approximately 200,000 of the NCI structures are not found in any of the other six analyzed databases. Therefore, the NCI "open" database provides a large number of unique synthetic compounds and natural products and is an excellent resource for drug lead discovery.

Using the modeled 3D structure of Bcl-2, we searched the NCI 3D-database of 250,000 small molecules using the program DOCK. In the database search, conformational flexibility of the small molecules was taken into account. The small molecules were ranked according to their scores as calculated using the energy scoring function in the DOCK program. The top 500 candidate small molecules with the best scores were considered as potential Bcl-2 inhibitors. Only non-peptide molecules were selected for testing. We have obtained samples of more than 150 compounds from the National Cancer Institute and tested their binding affinity to Bcl-2 using a sensitive and quantitative *in vitro* fluorescence polarization (FP) based binding assay.

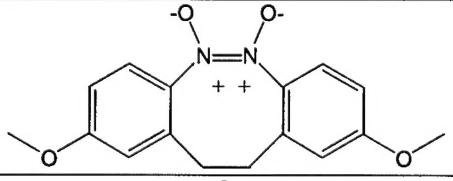
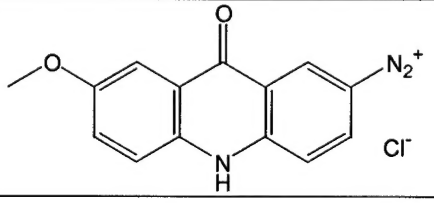
Task 2. *In vitro* biological confirmation of potential Bcl-2 inhibitors and mechanism investigations (**3-30 months**).

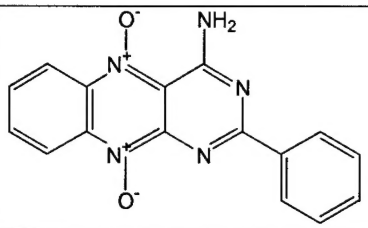
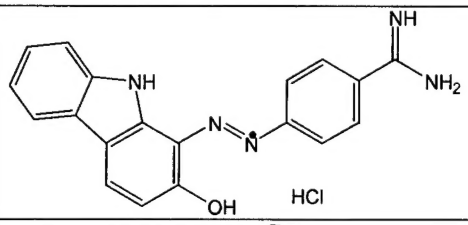
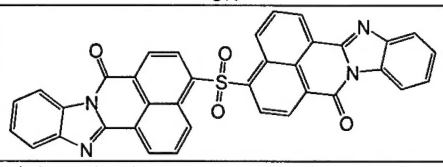
Task 2.1. Testing of potential small molecule inhibitors of Bcl-2 using an *in vitro* fluorescence polarization (FP) based binding assay.

We have used a sensitive and quantitative *in vitro* fluorescence polarization (FP) based binding assay to test the binding of these potential small molecule inhibitors to Bcl-2. The basic principle behind this assay is competition: a fluorescent peptide tracer (Flu-Bak-BH3) and a non-fluorescent small molecule inhibitor compete for binding to the target protein (Bcl-2). In a reaction mixture containing no small molecule inhibitor, the fluorescent tracer (Flu-Bak-BH3) is bound to the target protein (Bcl-2) and the emission signal is polarized; however, in a reaction mixture containing a small molecule inhibitor, the tracer is displaced by the small molecule inhibitor from the target protein and the emission signal becomes depolarized. The resulting change in the FP signal is directly related to the inhibitory activity of the small molecule inhibitor.

The binding affinity of these 150 candidate small molecules from the NCI's 3D-database was tested initially at a dose of 100 μM in this binding assay. Of which, 30 compounds showed inhibitory activity more than 50% at the initial 100 μM dose level and were classified as active. Further dose dependent binding experiments showed that most of these 30 compounds displayed a dose dependent inhibition of the Bak peptide binding to Bcl-2. The chemical structures and IC_{50} values of 5 representative small molecule inhibitors are shown in Table 1.

Table 1. Chemical structures and binding affinities of representative small molecule inhibitors of Bcl-2 discovered in this project.

	Chemical structure	Binding affinity to Bcl-2 (IC_{50} μM)
1 (BL-11)		10.4 ± 0.3
2		10.4 ± 1.2

3		1.6 ± 0.1
4		5.8 ± 2.2
5		7.7 ± 4.5
6 (BL-106)	(chemical structure not shown)	0.6 ± 0.1

As can be seen from Table 1, these active compounds have an IC_{50} value better than 20 μM. Compound **3** is the most potent compound in the binding assay, with an IC_{50} value of 1.6 μM. The other active compounds have an IC_{50} value from 5.8 to 10.4 μM. It is of note that these active compounds belong to different chemical classes and their structures are also different from other reported Bcl-2 inhibitors.

Our results thus suggest that computational structure-based 3D-database screening is quite effective for the discovery of small molecule inhibitors of Bcl-2.

Most recently, we have discovered several new small molecule inhibitors. One such compound (**BL-106**) has an IC_{50} value of 600 nM to Bcl-2 protein in our FP-based binding assay (**Table 1**). We further showed that **BL-106** binds to the BH3 binding pocket in Bcl-2 and Bcl-xL proteins using NMR methods (data not shown). Thus, **BL-106** represents a potent and promising Bcl-2 small-molecule inhibitor.

Task 2.2. Testing the activity of small molecule inhibitors of Bcl-2 in human breast cancer cells.

Binding experiments showed that these active compounds are capable of competing with the Bak BH3 peptide binding to Bcl-2 *in vitro*. Since we are interested in identifying cell permeable Bcl-2 small molecule inhibitors, we have tested the inhibitory activity of these active compounds on cell viability and proliferation using two different assays. First, the trypan blue exclusion method was used to determine the effect of an inhibitor on cell viability in which cells

were treated with the inhibitor for 24 hours. Second, the MTT assay was used to determine the activity of an inhibitor on cell proliferation where cells were treated for four days. It is important to keep in mind that an active compound in the binding assay could fail to show any cellular activity simply because of its poor cellular permeability. We first tested these active compounds using the HL-60 cell line. HL-60 is a human myeloid leukemia cell line and expresses the highest level of Bcl-2 protein among all the cancer cell lines examined in our laboratories. We then tested these active compounds using human breast cancer cell lines with different levels of Bcl-2 protein expression.

Using the trypan blue exclusion assay, these compounds were screened for their activity in inhibition of cell viability. More than half of the active compounds in the binding assay also have quite potent activity in cancer cells. For example, compound **1** is the most potent compound in the cellular assay, with an IC_{50} value of 10 μM. We have further tested compound **1** for its ability to inhibit cell growth. In the MTT assay where cells were treated for 4 days, **1** showed a potent inhibition in cell growth with an IC_{50} value of 4 μM. It was found that compound **1** potently inhibits cell growth in human breast cancer cell lines MDA-MB-231 with a high level of Bcl-2 (**Figure 2**). Compound **1** only has a weak activity in human breast canner cell lines MDA-MB-453 and T-47 with low levels of Bcl-2 protein. Therefore, compound **1** has selectivity between cancer cell lines with high level of Bcl-2 and low levels of Bcl-2 It is predicted that a potent small molecule that binds to the BH3 binding site of Bcl-2 will block the anti-apoptotic function of Bcl-2, which in turn would induce apoptosis in cancer cells with Bcl-2 protein overexpression. To test this hypothesis, we evaluated the ability and importantly the specificity of **1** in inducing apoptosis in cancer cells with high or low level of Bcl-2 expression. We used the Annexin-V flow cytometry assay to obtain a quantitative assessment on the ability of **1** in induction of apoptosis in HL-60 and human breast cancer MDA-231 cell line. MDA-MB-231 cells treated with 0 (untreated), 5 and 10 μM of compound **1** for 24 hours exhibited 0, 13% and 20.0 % apoptotic cells, respectively, while HL-60 cells treated with 0, 5, 10 and 20 μM of **1** for 24 hours had 0, 24%, 31% and 67% of apoptotic cells, respectively. Therefore, compound **1** induced apoptosis in a dose-dependent manner in MDA-MB-231 and HL-60 cell lines with Bcl-2 protein overexpression. In the human breast cancer cell line MDA-MB-453 and the normal fibroblast cell line WI-38 with low levels of Bcl-2 protein, no significant apoptotic cells were detected at 20 μM of compound **1**.

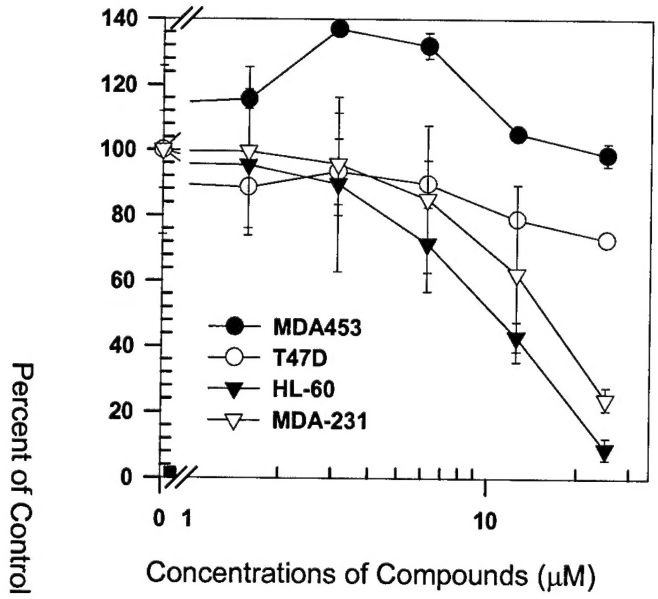


Figure 2. Inhibition of cell growth of compound **1** in human breast cancer and other cancer cell lines.

We have also tested the activity of **BL-106** in inhibition of cell growth using the MTT assay (**Figure 3**). As can be seen, the IC_{50} value in inhibition of cell growth in HL-60 cells are 0.7 μM, 5-times more potent than compound **1 (BL-11)**. In breast cancer MDA-231 (high Bcl-2) and MDA-453 (low Bcl-2) cell lines, the IC_{50} values are 1.6 and 6.5 μM, respectively. Thus, similar to compound **1** (**BL-11**), the activity of **BL-89-4** in inhibition of cellular growth correlates with the level of Bcl-2 protein in these cell lines. But **BL-89-4** is 5-times potent in cells than **BL-11**.

Based upon the binding and cellular activity, it is predicted that **BL-106** will be more potent than **BL-11** in induction of apoptosis. Indeed, when HL-60 cells were treated with 1.25, 2.5 and 5 μM of **BL-106** for 24 hrs, 19, 69 and 88% of cells underwent apoptosis. When MDA-231 cancer cells were treated with 5 μM of **BL-106,** 48% of cells underwent apoptosis. If the apoptotic effect in cells by **BL-106** is due to its ability to block the Bcl-2 anti-apoptotic function, this effect would depend upon the level of Bcl-2 expression. To test this hypothesis, we then tested the ability of **BL-106** in MDA-453 with low Bcl-2 expression. When MDA-453 cancer cells were treated with 5

μM of **BL-89-4**, less than 5% of cells underwent apoptosis. Thus, the ability of **BL-106** in inducing apoptosis in cancer cells correlates with the level of Bcl-2 expression. Furthermore, **BL-106** is much more potent than **BL-11** in induction of apoptosis, consistent with the binding affinity and activity in inhibition of cell growth.

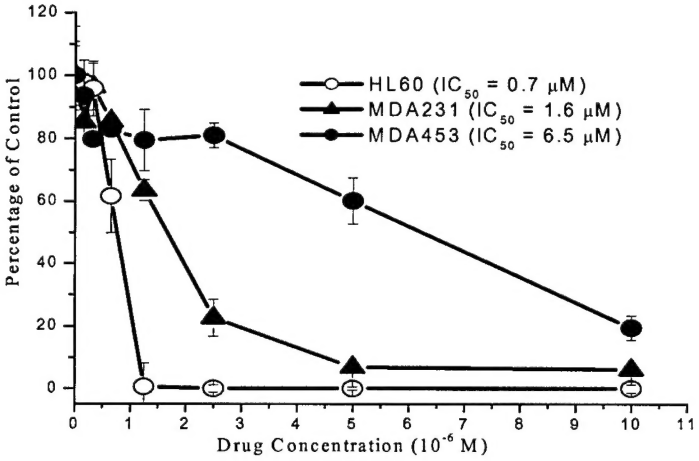


Figure 3. Inhibition of cell growth in human breast cancer cells (MDA-MB-231 and MBA-MB-453) and HL-60 cells by **BL-106.**

Task 3. *In vivo* testing of 2-3 most promising lead compounds (**24-36 months**).

Based upon our data, **BL-106** appears to represents a promising Bcl-2 small molecule inhibitor. To further test its therapeutic potential for the treatment of human breast cancer with Bcl-2 overexpression, we have synthesized 2 grams quantity of **BL-106**. We plan to perform extensive *in vivo* anti-tumor activity of **BL-106** using MDA-MB-231 xenograft model of human breast cancer in the next 6-12 months.

Task 4. Preparing scientific publications (**6-36 months**).

We are currently completing 2 manuscripts, disclosing the discovery of structurally diverse and potent small molecule inhibitors of Bcl-2. These manuscripts will be submitted for publication within the next 6-12 months.

Key Research Accomplishments:

(1). We have discovered more than 6 chemical classes of novel small-molecule inhibitors of Bcl-2. One small-molecule inhibitor BL-106 displays a very potent binding affinity to the Bcl-2 protein in our binding assay.

(2). We have evaluated the activity and selectivity of these promising Bcl-2 small-molecule inhibitors in human breast cancer cells and other cancer cells. We found that small-molecule inhibitors **BL-11** (compound **1**) and **BL-106** (compound **6**) potently inhibit cell growth in cancer cells with high levels of Bcl-2 protein and have selectivity in cancer cells with low levels of Bcl-2 protein. In fact, **BL-106** is a very potent, cell-permeable, non-peptidic small molecule inhibitor.

(3). In apoptosis-assay, **BL-106** potently induces apoptosis in MDA-MB-231 breast cancer cells and has little activity in MDA-MB-453 breast cancer cells with low levels of Bcl-2 protein.

(4). Based upon our results, we have developed a synthetic method for BL-106 and have synthesized large quantity for in vivo anti-tumor activity studies in the MDA-MB-231 xenograft model of human breast cancer.

Reportable Outcomes:

(1). A manuscript described the discovery of **BL-106** and other novel small-molecule inhibitors is currently being prepared and will be submitted shortly.

(2) An oral presentation was made in the 2002 DOD Era of Hope Meeting in Orlando, Florida. This presentation was well-received by both scientists and breast cancer advocates.

(3). An invention disclosure has been filed with University of Michigan Technology Transfer Office to disclose the discovery of BL-106 and other novel small-molecule inhibitors of Bcl-2.

Conclusions: Although this Award was only received in July, 2003 due to the transfer of the grant from Georgetown University to University of Michigan, we have well on our way to accomplish all the major goals and tasks we proposed in our original proposals. More than 6 classes of structurally diverse, non-peptidic, drug-like, small-molecule inhibitors of Bcl-2 have been successfully identified. Our cellular studies also showed that **BL-11** and **BL-106** inhibited cell growth and induced apoptosis in cancer cells with high levels of Bcl-2 proteins and displayed selectivity in cancer cells with low levels of Bcl-2 proteins. We have developed an efficient synthetic method for BL-106 and synthesized large quantity for in vivo studies. A manuscript is about to be completed and a patent disclosure has been filed. The oral presentation made in the DOD Era of Hope meeting on this project was well received.